

Bovine Herpesvirus 1 VP22 Enhances the Efficacy of a DNA Vaccine in Cattle†

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For this study, the intercellular trafficking ability of bovine herpesvirus 1 (BHV-1) VP22 was applied to improve the efficacy of a DNA vaccine in calves. A plasmid encoding a truncated version of glycoprotein D (tgD) fused to VP22 was constructed. The plasmid encoding tgD-VP22 elicited significantly enhanced and more balanced immune responses than those induced by a plasmid encoding tgD. Furthermore, protection against a BHV-1 challenge was obtained in calves immunized with the plasmid encoding tgD-VP22, as shown by significant reductions in viral excretion. However, less significant protection was observed for animals vaccinated with the tgD-expressing plasmid, correlating with the lower level of immunity observed prechallenge. This is the first report of the use of VP22 as a transport molecule in the context of a DNA vaccine for a large animal species.

Bovine herpesvirus 1 (BHV-1) causes a variety of clinical manifestations and may predispose animals to secondary bacterial infections (34). Vaccination with conventional live attenuated or inactivated vaccines has been the predominant control strategy against BHV-1. In addition, although they are not commercially available, a subunit vaccine using a truncated version of glycoprotein D (tgD) and a vaccine with a deletion of gE have been developed (3, 21, 31). Although live vaccines are thought to induce higher levels of protective immunity, they may cause abortions or clinical disease if they are insufficiently attenuated (32). Modified live vaccines can also become latent, with subsequent reactivation and shedding. Most importantly, the available vaccine strains, like the wild-type virus, may down-regulate the cell surface expression of major histocompatibility complex class I molecules (12, 23), which likely compromises the development of cytotoxic T lymphocytes against not only BHV-1, but also other viruses and intracellular pathogens. Killed vaccines may not provide complete protection due to a low antigen load or a loss of important epitopes during inactivation, and they generally are poor inducers of cellular immunity. Another disadvantage of killed vaccines is the relatively short duration of immunity (7).

DNA vaccines have emerged as an attractive approach for the generation of antigen-specific immunity, both for humans and for veterinary species. However, the potency of naked DNA vaccines is limited by their inability to amplify and spread in vivo. Thus, although DNA vaccines are generally very effective in mouse models, several challenges must be overcome for their use in large outbred species (2). BHV-1 VP22 is a 258-amino-acid tegument protein (18) which can transport proteins from the cells in which they were originally produced to neighboring cells (13). A major hurdle to DNA vaccination is the

small number of cells that are transfected, but this may be overcome in part by utilizing the intercellular trafficking capacity of VP22 to disseminate the expressed antigen to neighboring cells, thus increasing antigen presentation.

Herpes simplex virus type 1 (HSV-1) VP22 has been defined as a member of the so-called ferry proteins, since there is evidence that HSV-1 VP22 traffics from a transfected cell to neighboring cells, where the protein is translocated through a nonclassic, undefined mechanism (9). Although attempts to detect the ability of intercellular trafficking of HSV-1 VP22 in live cells have been unsuccessful (1, 9, 10, 20), HSV-1 VP22 has been successfully used to deliver p53 or thymidine kinase into cells in vitro through intercellular spreading, where these proteins exhibit their natural functions (8, 25, 33). Furthermore, VP22 proteins from both HSV-1 and Marek's disease virus have been shown to enhance cell-mediated immune responses in mice when expressed from plasmids as fusion proteins with human papillomavirus type 16 E7 (14, 17, 22). The immunization of mice with a plasmid encoding yellow fluorescent protein (YFP) fused to BHV-1 VP22 stimulated immune responses superior to those elicited by standard DNA immunization (24). However, the efficacy of VP22 as a transporter molecule in a large animal model has not been evaluated yet.

For this study, our objective was to determine whether a plasmid encoding BHV-1 tgD fused to VP22 could elicit an enhanced immune response in a large animal species such as cattle compared to the response elicited by a plasmid encoding tgD alone. To confirm the intercellular trafficking property of VP22 in the context of tgD-VP22, we constructed the plasmids pVP22-YFP, pMASIA-tgD-YFP, and pMASIA-tgD-VP22-YFP. For the construction of pVP22-YFP, the U_L49 (VP22 gene) open reading frame was amplified from BHV-1 genomic DNA by PCR and then inserted into pEYFP-N1 (Clontech, BD Biosciences, Palo Alto, Calif.). Subsequently, pMASIA-tgD-YFP and pMASIA-tgD-VP22-YFP were generated by PCR cloning of the YFP and VP22-YFP genes from pEYFP-N1 and pVP22-YFP, respectively, into pMASIA-tgD,

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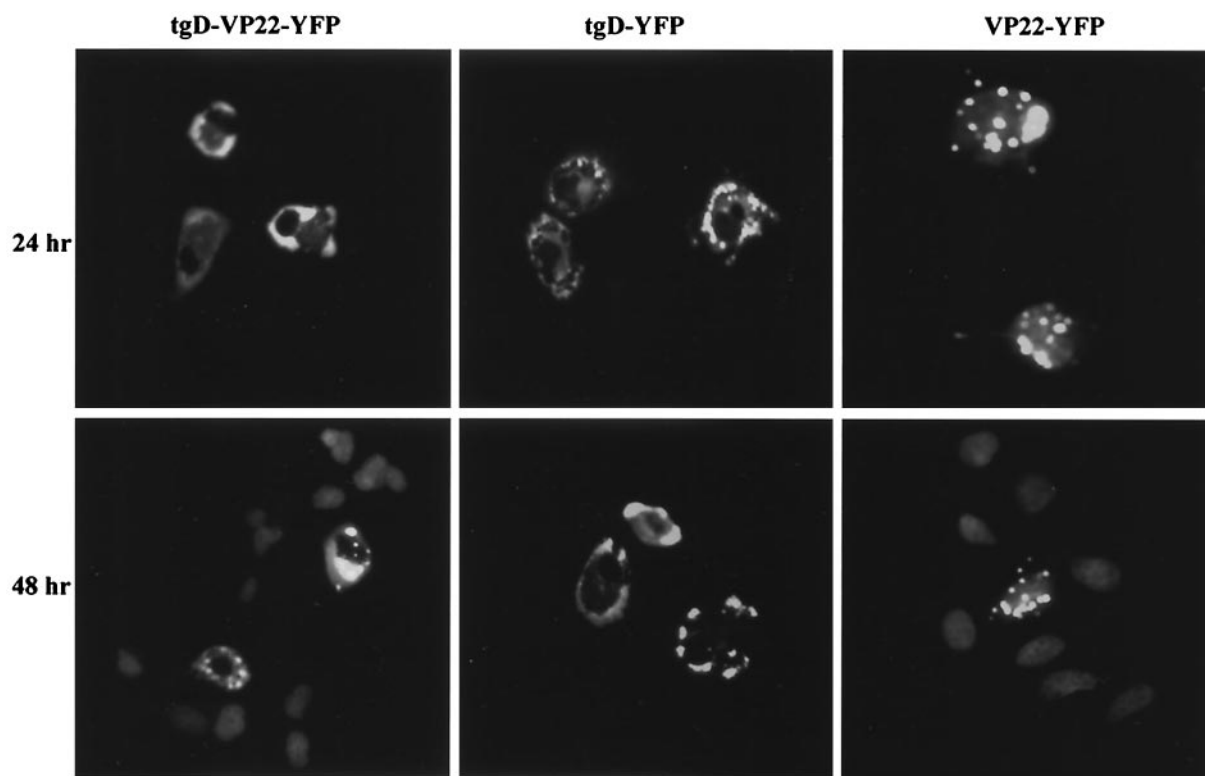


FIG. 1. Inter-cellular trafficking of tgD-VP22-YFP and VP22-YFP in live transfected cells. COS-7 cells were transfected with a plasmid expressing tgD-VP22-YFP, tgD-YFP, or VP22-YFP. Live cells were examined by fluorescence microscopy 24 and 48 h after transfection. Magnification, $\times 40$.

which encodes BHV-1 tgD (26). The apparent molecular masses of tgD-VP22-YFP, tgD-YFP, and VP22-YFP were confirmed by Western blot analysis to be 124, 89, and 63 kDa, respectively (data not shown). COS-7 cells were transfected with pMASIA-tgD-VP22-YFP, pMASIA-tgD-YFP, and pVP22-YFP and monitored every 4 h by fluorescence microscopy. No inter-cellular transport was observed during the first 24 h after transfection (Fig. 1). However, like pVP22-YFP-transfected cells, pMASIA-tgD-VP22-YFP-transfected cells were surrounded by increasing numbers of cells with exclusive nuclear staining after this time interval. As time progressed, the fluorescence of the transported VP22-YFP became stronger, and at 48 h after transfection, inter-cellular trafficking was obvious (Fig. 1). This time point is consistent with previous observations of inter-cellular trafficking of BHV-1 VP22 (13) and HSV-1 VP22 when analyses were performed on fixed cells (1, 4). Indeed, we counted five fields of 100 cells each at 0, 24, 36, and 48 h posttransfection with pMASIA-tgD-VP22-YFP and observed that at 36 and 48 h, $45.6\% \pm 8.3\%$ and $78.2\% \pm 11.3\%$ of the transfected cells, respectively, were surrounded by cells with exclusive nuclear staining.

To test the adjuvant effect of BHV-1 VP22 in the context of a DNA vaccine, we amplified the full-length open reading frame of VP22 (18) by PCR and inserted it into pMASIA-tgD (26). This created pMASIA-tgD-VP22, which encoded tgD fused in frame to VP22. Western blot analyses of pMASIA-tgD-VP22- and pMASIA-tgD-transfected COS-7 cells demonstrated that tgD-VP22 and tgD had apparent molecular masses

of 96 and 61 kDa, as predicted (data not shown). This suggested that tgD was properly folded and glycosylated in the secretory pathway. Hereford calves were immunized intradermally with 500 μ g of pMASIA-tgD-VP22, 500 μ g of pMASIA-tgD (26), or saline in a 500- μ l volume by use of the Biojector 2000 needle-free injection system (Bioject, Bedminster, N.J.). All animals were reimmunized after 28 days and were challenged with BHV-1 strain 108 (28) on day 54. All data from this study were analyzed with the aid of Graphpad Prism 3.0. Prior to analyses, data that were not normally distributed were transformed by log transformation. Differences between the vaccine groups were examined by one-way analysis of variance for the indicated time points. If the overall test result for a treatment effect was significant by one-way analysis of variance, then the means of the transformed variables were compared by Tukey's method.

Immunization with either pMASIA-tgD or pMASIA-tgD-VP22 resulted in significant tgD-specific immunoglobulin G (IgG) titers in sera ($P < 0.001$) (Fig. 2a). Moreover, the IgG titers of pMASIA-tgD-VP22-immunized calves were higher than those of pMASIA-tgD-immunized animals after both primary ($P < 0.05$) and secondary ($P < 0.001$) immunization. After the challenge, both plasmid-vaccinated groups displayed higher anamnestic responses ($P < 0.001$) than the control group, and the pMASIA-tgD-VP22 group had higher IgG titers than the pMASIA-tgD group ($P < 0.001$). Furthermore, compared to the pMASIA-tgD group, the pMASIA-tgD-VP22 group had lower ($P < 0.05$) IgG1-to-IgG2 ratios both before

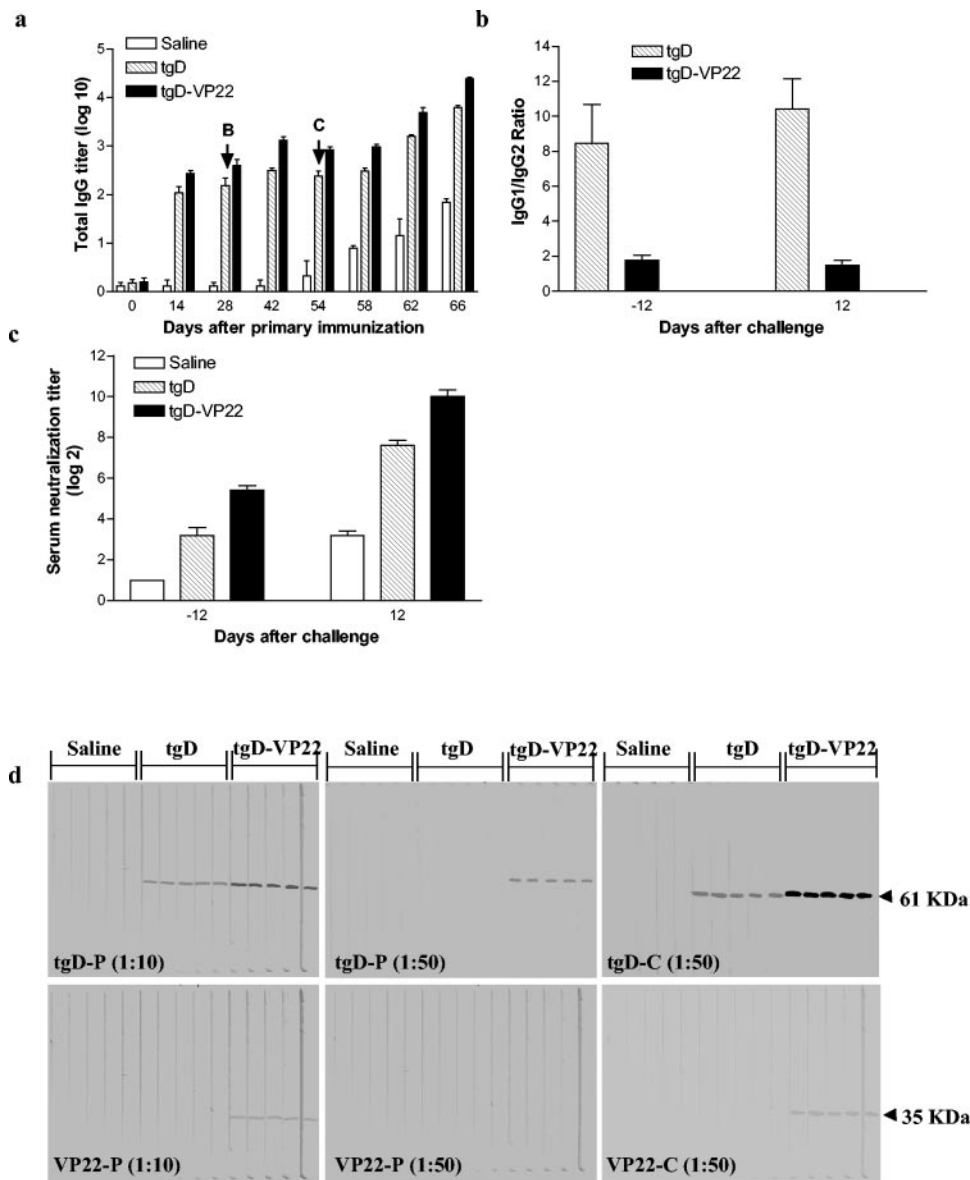


FIG. 2. Antigen-specific serum antibody responses. Three groups ($n = 5$) of calves were injected intradermally with saline, pMASIA-tgD, or pMASIA-tgD-VP22 on day 0 and then given a booster (B) on day 28. All animals were challenged (C) on day 54. (a) Mean tgD-specific IgG titers \pm standard errors of the means (SEM), determined by ELISA as previously described (30). The titers were calculated as reciprocal serum dilutions at which the optical density reading was two standard deviations above the value of a negative control serum. (b) Serum isotype ratios on day 12 before and day 12 after challenge. Results are shown as average ratios \pm SEM. (c) Virus neutralization titers \pm SEM on day 12 before and day 12 after challenge. The virus neutralization titers in serum samples were determined as previously described and were expressed as reciprocals of the highest dilutions of antibody that caused a 50% reduction in the number of plaques relative to the virus control of 100 PFU of BHV-1 (30). (d) Analysis of tgD- and VP22-specific serum antibody responses by Western blotting. Culture supernatants from pMASIA-tgD-transfected Cos-7 cells and cell lysates from pMASIA-VP22-transfected Cos-7 cells were transferred to nitrocellulose following separation in a 10% reducing gel and then were incubated with bovine sera collected 12 days prior to (P) and 12 days after (C) challenge. The sera were derived from animals that had been vaccinated with saline, pMASIA-tgD, or pMASIA-tgD-VP22 and were tested at either a 1:50 dilution or a 1:10 dilution. The reactions were visualized with alkaline phosphatase-conjugated rabbit anti-bovine IgG (Kirkegaard and Perry Laboratories) and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad Laboratories). The positions of tgD (61 kDa) and VP22 (35 kDa) are indicated.

and after challenge (Fig. 2b). The virus neutralization titers agreed with enzyme-linked immunosorbent assay (ELISA) titers, with both plasmid-vaccinated groups being significantly different from the control group ($P < 0.001$) (Fig. 2c). In addition, the pMASIA-tgD-VP22 group had higher neutralization titers ($P < 0.001$) than the pMASIA-tgD group. A West-

ern blot confirmed that prior to challenge, the pMASIA-tgD-VP22 group developed a stronger tgD-specific humoral response than the pMASIA-tgD group (Fig. 2d). Furthermore, low VP22-specific antibody levels were detected for the pMASIA-tgD-VP22 group. After challenge, both tgD- and VP22-specific reactions became stronger.

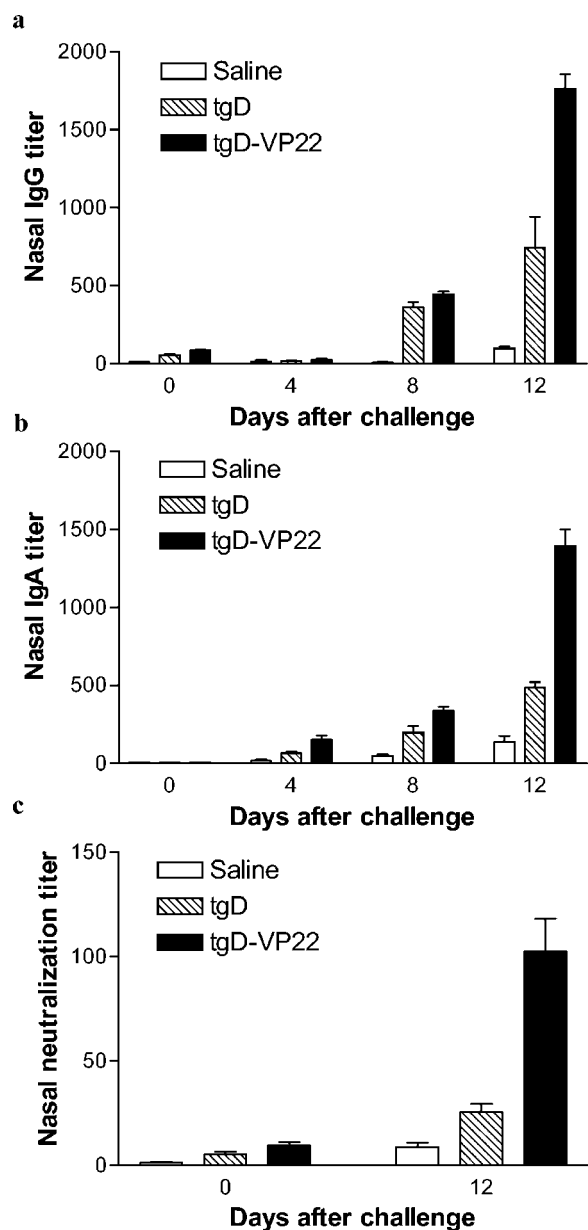


FIG. 3. gD-specific nasal antibody responses. Three groups ($n = 5$) of calves were injected intradermally with saline, pMASIA-tgD, or pMASIA-tgD-VP22 as described in the legend to Fig. 1. (a) Mean tgD-specific IgG titers \pm SEM, determined by ELISA as previously described (19). (b) Mean tgD-specific IgA titers \pm SEM, determined by ELISA (19). Titers are expressed as reciprocals of the highest dilutions resulting in a reading of 2 standard deviations above the control value. (c) Virus neutralization titers in nasal fluids before and on day 12 after challenge, expressed as reciprocals of the highest dilutions of antibody that caused a 50% reduction in the number of plaques relative to the virus control of 100 PFU of BHV-1 (8, 30).

Prior to as well as after challenge, the tgD-specific nasal IgG titers of both plasmid-vaccinated groups were higher ($P < 0.01$) than those of the control group. There was also a significant difference between the pMASIA-tgD and pMASIA-tgD-VP22 groups, both before ($P < 0.05$) and 8 ($P < 0.05$) and 12 ($P < 0.01$) days after challenge (Fig. 3a). Although no tgD-

specific nasal IgA titers were detected prior to challenge, the nasal IgA titers of the plasmid-vaccinated groups were higher (for pMASIA-tgD, $P < 0.01$; for pMASIA-tgD-VP22, $P < 0.001$) than those of the control group after challenge (Fig. 3b). Furthermore, the nasal IgA titers of the pMASIA-tgD-VP22 group were higher than those of the pMASIA-tgD group on days 4 ($P < 0.05$), 8 ($P < 0.05$), and 12 ($P < 0.001$). Before the challenge, low neutralizing antibody levels were measured in the nasal fluids, with the only significant difference occurring between the pMASIA-tgD-VP22 group and the saline group ($P < 0.01$) (Fig. 3c). By day 12 postchallenge, the pMASIA-tgD-VP22 group had higher nasal neutralizing antibody titers than the saline and pMASIA-tgD groups ($P < 0.001$).

To further analyze the immune responses induced by the two different constructs, we determined the lymphocyte proliferative responses (19). After primary as well as secondary immunization, the tgD-specific proliferative responses of both plasmid-vaccinated groups were superior to those of the control group ($P < 0.001$). Furthermore, the pMASIA-tgD-VP22 group had higher stimulation indexes (SIs) ($P < 0.05$) than the pMASIA-tgD group (Fig. 4a). All groups exhibited increased tgD-specific proliferative responses after challenge, with both the pMASIA-tgD- and pMASIA-tgD-VP22-vaccinated groups having higher SIs ($P < 0.01$ and 0.001 , respectively) than the control group and with the pMASIA-tgD-VP22 group having higher SIs ($P < 0.05$) than the pMASIA-tgD group. To confirm T-cell activation, we assessed tgD-specific gamma interferon (IFN- γ) production by using enzyme-linked immunospot assays (Fig. 4b) (27). Both vaccinated groups had larger ($P < 0.001$) numbers of IFN- γ -secreting cells than the control group, with the pMASIA-tgD-VP22 group having larger numbers than the pMASIA-tgD group after both primary ($P < 0.05$) and secondary ($P < 0.01$) immunization. Following challenge, the numbers of tgD-specific IFN- γ -secreting cells increased in all animals. Nevertheless, there was a significant difference in the numbers of IFN- γ -secreting cells between the plasmid-vaccinated and control groups ($P < 0.001$) as well as between the pMASIA-tgD-VP22 and pMASIA-tgD groups ($P < 0.01$). When this experiment was repeated essentially in the same manner, but with phosphate-buffered saline as the solvent for the plasmids, we again observed significantly enhanced immune responses for the pMASIA-tgD-VP22 group compared to the pMASIA-tgD group (data not shown).

On the day of challenge and for up to 12 days afterwards, all animals were monitored to evaluate the protection achieved by immunization. Both plasmid-immunized groups shed less virus ($P < 0.01$) than the saline group from day 6 onwards, and the pMASIA-tgD-VP22 group displayed less shedding than the pMASIA-tgD group on days 2 ($P < 0.05$), 4 ($P < 0.05$), and 6 ($P < 0.001$) (Fig. 5). All animals experienced weight loss and had increased rectal temperatures. However, there were no significant differences between the groups, which may be due to the relatively small group sizes.

This is the first report describing the potential of BHV-1 VP22 as a transport molecule in the context of a DNA vaccine for a large, outbred animal. In contrast to the antigens tested with HSV-1 VP22 (5, 14, 15), BHV-1 tgD is a very strong immunogen which is secreted from transfected cells. The observation that tgD became more efficient when fused to BHV-1 VP22 confirmed that tgD retained its antigenic structure and

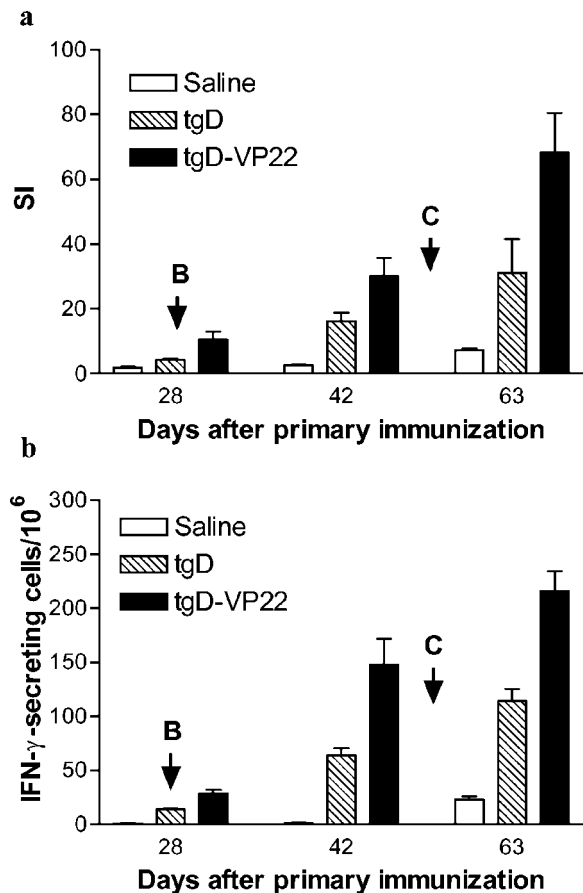


FIG. 4. gD-specific cellular immune responses. Three groups ($n = 5$) of calves were injected intradermally with saline, pMASIA-tgD, or pMASIA-tgD-VP22 as described in the legend to Fig. 1. (a) Antigen-specific lymphocyte proliferation. Peripheral blood mononuclear cells were isolated on Ficoll-Paque Plus as previously described (19) and then stimulated with purified gD at 1 μ g/ml or cultured in medium alone. After 72 h in culture, the cells were pulsed with 0.4 μ Ci of [methyl- 3 H]thymidine/well. The cells were harvested 18 h later, and radioactivity was determined by scintillation counting. Proliferative responses were calculated as the means of triplicate wells and are expressed as SIs, which represent counts per minute in the presence of gD divided by the counts per minute in the medium alone. The values are averages \pm SEM of the SIs. (b) Mean numbers of tgD-induced IFN- γ -secreting cells in blood \pm SEM, determined as previously described (19). Peripheral blood mononuclear cells were cultured in IFN- γ -coated plates containing medium alone or 0.4 μ g of gD per ml. The numbers of IFN- γ -secreting cells were expressed as the differences between the numbers of spots per 10^6 cells from gD-stimulated cultures and the number of spots per 10^6 cells from medium-only control cultures.

that VP22 was functional, even though it passed through the secretory pathway in cells. Although it is conceivable that priming of a VP22-specific antibody response had some impact on protection, there was no response at the time of challenge (Fig. 2) and there is no evidence that VP22 is a protective antigen. Thus, the enhanced protection of the pMASIA-tgD-VP22 group correlated with and was likely due to improved tgD-specific immune responses.

We have previously demonstrated a strong correlation between gD-specific neutralizing antibody titers at the time of

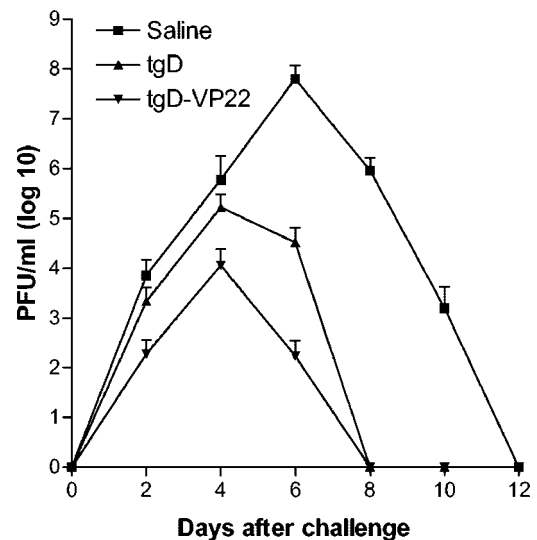


FIG. 5. Virus shedding after challenge with BHV-1. Calves were immunized as described in the legend to Fig. 1 and then challenged with a 4-min aerosol of 10^7 PFU of BHV-1 strain 108/ml 26 days after secondary immunization as previously described (19). Virus was recovered from the nasal fluids and quantified by plaque titration in microtiter plates with an antibody overlay as described previously (19). All data are means for each group \pm SEM.

BHV-1 challenge and reductions in virus shedding after challenge (31). In another trial, we showed that the induction of a more balanced tgD-induced immune response without enhanced neutralizing antibodies did not result in enhanced protection from a BHV-1 challenge (16). Furthermore, a shift in the type of immune response towards enhanced T-cell responses did not correlate with enhanced protection (19, 26). These observations further suggest that even if VP22 did have T-cell epitopes, which might contribute to the cellular response, the enhanced tgD neutralizing antibody response was an important factor in the improved protection observed for the pMASIA-tgD-VP22 group.

In contrast, for many pathogens, cell-mediated immunity, and not the presence of an antibody, is correlated with protection (6, 16). In these situations, a shift towards a Th2 bias induced by plasmid-encoded secreted antigens in contrast to their cell-associated counterparts (11, 29) may reduce the efficacy of the DNA vaccine. Interestingly, whereas intradermal delivery of a plasmid encoding tgD elicited predominantly IgG1, as expected, the immunization of calves with a plasmid encoding tgD-VP22 resulted in more balanced immune responses. This is in agreement with previous reports, which suggested that VP22 causes a switch in the immune response from a Th2-type to a Th1-type bias in mice (24). This ability of VP22 to shift the response to be balanced or biased towards Th1 is of considerable importance for those pathogens against which cell-mediated immunity is required for protection from infection.

Currently, the mechanism by which VP22 can enhance and shift the immune response to antigens that are expressed in vivo is not clear. VP22 may improve immune presentation in different ways, either through enhanced direct priming by transfected antigen-presenting cells (APCs) or by amplified

cross-presentation after release from transfected nonprofessional APCs (22). Indeed, in one study, an increased number of APCs was observed in the draining lymph nodes of mice that received the VP22 fusion protein (17).

In conclusion, these data clearly demonstrate that BHV-1 VP22 can be used as a transport molecule in the context of a DNA vaccine to enhance both humoral and cellular immune responses in a large animal species. Furthermore, a tgD-VP22 DNA vaccine induced a stronger and more balanced immune response than a tgD DNA vaccine. The ability of VP22 to balance the immune response is an important characteristic that may be utilized to induce protection against a variety of viral and intracellular bacterial pathogens.

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